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Title:

Transforming growth factor (TGF) β 1 and Smad signaling pathways: A likely key to EMT- associated COPD pathogenesis

Short Title: Airway EMT: TGF β ₁-pSmad pathway

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Abbreviations list

COPD:	Chronic Obstructive Pulmonary Disease
EMT:	Epithelial Mesenchymal Transition
GOLD:	Global Initiative for Chronic Obstructive Lung Disease
TGF:	Transforming Growth Factor
FEV ₁ :	Forced Expiratory Volume in 1 second
FVC:	Forced Vital Capacity
Rbm:	Reticular Basement Membrane
LP:	Lamina Propria
ECM:	Extra Cellular Matrix
TNF:	Tumor necrosis Factor
IFN:	Interferon
H-N:	Healthy Non Smoker
NLFS:	Normal lung function smoker
S-COPD:	Smoking COPD
ES-COPD:	Ex-Smoker COPD

Summary at glance

COPD is fundamentally due to small airway fibrosis in association with exposure to cigarette smoke. Pathologically, it is associated with active EMT whereby epithelial cells develop mesenchymal phenotype and become invasive. Relatively little has been known regarding the association of the TGF β ₁-pSmad pathway with EMT in smokers and COPD. This study facilitates the understanding of EMT-associated pathogenesis of COPD as a result of smoking induced intracellular downstream Smad transcriptional pathways.

Abstract

Background: COPD is characterised by poorly reversible airflow obstruction usually due to cigarette smoking. TGFβ₁ has been implicated in the pathogenesis of COPD, and in particular a process called epithelial mesenchymal transition (EMT), which may well be an intermediary between smoking and both airway fibrosis and lung cancer. The downstream classical or “canonical” TGFβ₁ pathway is via the phosphorylated (p) Smad transcription factor system.

Methods: We have investigated TGFβ₁ expression and its “pSmad fingerprint” in bronchoscopic airway biopsies from patients with COPD, and in smoking and non-smoking controls. A cross sectional immunohistochemical study compared TGFβ₁ and pSmad 2, 3 (excitatory) and 7 (inhibitory) expression in cells and blood vessels of 3 compartments of large airways: epithelium (especially the basal region), reticular basement membrane (Rbm) and underlying lamina propria (LP).

Results: TGFβ₁ expression was higher generally in COPD subjects throughout the airway wall (P<0.01), while pSmad 2/3 expression was associated with smoking but especially current smoking COPD (P<0.05). Expression of inhibitory pSmad-7 was also prominently reduced in patients with COPD in contrast to smokers and controls (P<0.01). In addition, pSmad but not TGFβ₁ expression, was related to airflow obstruction and a canonical EMT biomarker (S100 A4) expression.

Conclusion: Activation of the Smad pathway in the airways is linked to EMT activity and loss of lung function. The disconnect between TGFβ₁ and pSmad in terms of relationships to EMT activity and lung function, suggests that factors other or in addition to TGFβ₁ are driving the process.

Keywords: TGF β ₁, Smad 2/3, Smad 7, EMT, COPD.

Introduction

Chronic obstructive pulmonary disease (1) is a poorly reversible chronic, slowly progressive airway obstructive respiratory disease. It is mainly smoking-related and primarily constitutes small airway fibrosis and destruction, more generalized “chronic bronchitis” and in some but not all later development of emphysema. Approximately 50% of smokers develop COPD eventually (2, 3).

Smoking is associated with airway luminal innate inflammation but the change within the airway wall in susceptible individuals are more characterized by tissue remodelling. TGFβ₁ is a multifunctional cytokine which induces a number of biological processes including regulation of angiogenesis and ECM components and has been implicated as a driver of COPD airway pathology (2, 4, 5). The best described intracellular pathway for TGFβ₁ actions is the so called “canonical” cascade involving up-regulation and phosphorylation of Smad 2/3 (5, 6); one outcome of this is activation of a process called Epithelial-mesenchymal Transition (EMT) (7), which we have described as active in COPD (8, 9).

Smads are transcription factors (10), including stimulatory, receptor-activated Smads 1, 2, 3, 5, 8 and 9; (11), a common co-mediator Smad4 (12) and inhibitory Smads 6 and 7; (13). Binding of TGF-β₁ to its Type II receptor leads to formation of a receptor complex that phosphorylates the Smad-2/3 complex, which in turn interacts with a transporter Smad 4 for translocation to the nucleus. In the nucleus, the pSmad complex binds to a specific promotor region of target genes (14) (Fig-1). Inhibitory Smads act as an important brake on TGF-β₁ signaling by binding stably to TGF-β₁ receptors and/or by competing for the common mediator Smad4 (10). Smad 7 is activated (phosphorylated) by proinflammatory cytokines TNF-α and IFN-γ but is

transcriptionally regulated by TGF- β_1 itself, as well as Epidermal Growth Factor (EGF) and ultraviolet radiation (15). Springer and colleagues previously demonstrated reduced Smad 7 gene expression in bronchial biopsies of COPD patients (16), suggesting that lack of Smad 7 could be relevant to pathogenesis (17)

A preliminary study from our group suggested that TGF β_1 was indeed functionally active through pSmad 2/3 expression in COPD (18). The present study has taken this further and evaluated TGF β_1 expression and its associated downstream “Smad fingerprint” in airway biopsy material from COPD subjects and appropriate smoking and non-smoking controls. We hypothesized that: 1) TGF β_1 and pSmad 2/3 expression is increased in smokers but especially in COPD subjects, 2) pSmad 7's protective role is down regulated in a reciprocal way to Smad 2/3 activation. 3) There may be a relationship between activation of the TGF β_1 pathway and EMT and airflow obstruction.

MATERIALS AND METHODS

Ethics approval

The Tasmania Health & Medical Human Research Ethics Committee approved the study (EC00337). All subjects gave written, informed consent prior to participation.

Subjects

64 subjects were recruited through advertisement. Bronchial biopsies (BB) from 15 smokers with normal lung function (S-N), 17 S-COPD and 17 ES-COPD were compared with 15 H-N (Table 1). COPD was diagnosed according to GOLD criteria (1). Subjects with other respiratory diseases, a history of recent acute exacerbation of COPD were excluded from the study. COPD subjects were on PRN (as needed)

bronchodilators for the most part, and especially none were using systemic or inhaled corticosteroids.

Tissue Processing

Biopsies were first fixed in 10% neutral buffered formalin for 2 hrs. then in 50% ethanol before processing on a Leica ASP 200 tissue processor. Paraffin embedded sections of 3mm were cut for staining, separated by at least 50 microns and mounted on slides.

After removal of paraffin, sections were stained with either monoclonal antibody anti-TGFβ₁ (abcam ab 27969 clone TB1 at 1/16000 26 mg/ml- overnight at room temperature) after blocking with Dako serum block (X0909), phosphorylated (activated) Smad2/3 (pSmad2/3) (Santa Cruz SC-11769R at 1:100 for 1hour at room temperature) following heat retrieval using a Dako PT link with high pH solution K800421 at 95 degrees for 30 minutes and pSmad 7((Santa Cruz SC-101152 at 1:100 for 60 minutes at room temperature) In each run a section stained with immunoglobulin (Ig) G1-negative control (X0931 clone DAKGO1; Dako Cytomation) was included to ensure absence of false positive staining and a known lung tissue positive tissue control was run with each staining. Bound antibodies were elaborated by using horseradish peroxidase (HRP) conjugated DAKO Envision plus reagent (cat no. K4001, anti-mouse or K4003 anti-rabbit) and diaminobenzidine (DAB) for a brown colour resolution (cat. no. K3468; Dako Cytomation). Nuclei were counterstained using Mayers Haematoxylin and sections dehydrated through ascending grades of ethanol, cleared in xylene and mounted in permount. We have extensively used and published with these methods (8, 19)

Tissue section analysis and quantitation

All slides were coded and randomized to blind the person who did the measurements (MM). We randomly choose five good fields for measurement from each slide, for each of the biomarkers. Only areas with intact epithelium and LP and without tissue damage were selected for measurement. Measurements were performed by computer-assisted image analysis using microscopy at 40x magnification (Leica DM 2500, Microsystems, Germany), a Spot insight 12 digital camera (Spot imaging, USA) and Image Pro V5.1 software (Media Cybernetics, USA).

All biomarkers (TGF β ₁, pSmad 2/3, pSmad 7) were quantitated as percentage of epithelial area so stained, as number stained basal epithelial cells, as well as cells and vessels stained in the Rbm per mm of Rbm. In addition, pSmad 2/3 and pSmad 7 immuno-staining were also quantified in LP cells and LP vessels as % of total cells and vessels.

Statistical analysis

Since the data were non-normally distributed, the results for each marker are presented as the median and range. Non-parametric ANOVA (Kruskal-Wallis) was first used to detect any overall difference among study groups, followed by Dunn's multiple comparison test to specify which groups were different. Finally, the Mann-Whitney U test used to confirmed statistical difference between groups. This method minimized multiple comparisons but appropriate correction was made to each P value where these were done. Statistical analyses were performed using SPSS (statistics version 20.0, IBM Co, USA) for Windows 7.0 and a p-value of ≤ 0.05 was considered statistically significant.

RESULTS

TGFβ₁ expression (Fig 2&3)

Epithelium

A greater proportion of the cells in the basal layer of the large airway epithelium stained for TGFβ₁ in NLFS, S-COPD and ES-COPD in comparison to H-N control group (p<0.05), but with no significant difference between smoker/ COPD groups.

Rbm:

Rbm cells highly expressed TGFβ₁ in NLFS, S-COPD, and ES-COPD in comparison to H-N control group (P<0.005), but this was most marked in S-COPD i.e. there was both a smoking and extra COPD effect (P<0.005).

TGFβ₁ expression on blood vessels in the Rbm was significantly increased in all smoker/COPD groups (p<0.05). The data were also analysed as percent of vessels that stained, since vessels numbers are markedly increase in this compartment in smokers/COPD groups. (20) The message was much the same when plotted as percentages.

LP:

There was either no or just light staining in a diffuse pattern in the normal controls throughout the LP, which was very much heavier, and uniformly so, in all smoker/COPD groups. This was not possible to quantify precisely.

pSmad 2/3 expression: (Fig 4&5)

Epithelium:

Compared to normal control (H-N), there was an increased proportion of cells in the basal area staining for pSmad 2/3 expression in smoking groups (both NLFS and S-COPD) ($P < 0.05$) but not in the ES-COPD group.

Rbm:

There was significantly increased expression of pSmad 2/3 in NLFS but especially in S-COPD. An elevated expression of pSmad 2/3 in Rbm cells in the ES-COPD group was intermediate between controls and the other pathological groups. i.e. there was predominantly a smoking effect but again some extra COPD effect.

Similarly, pSmad 2/3 vessel expression was greater in the NLFS and S-COPD groups, but not in the ES-COPD group. When these data were examined in terms of percent vessels, to take into account between-group variations, the outstanding feature was a marked increase in the S-COPD group.

LP:

In LP cells there was an increase pSmad 2/3 expression only in the COPD groups, perhaps a little more marked in S-COPD ($P < 0.001$). Total cell number in the LP are decreased in COPD, so data were analysed as percentage of cells expressing pSmad 2/3; percent cell staining was especially marked in the S-COPD group.

In the LP vessels, there was an increase in pSmad 2/3 expression in both smoking groups, but again especially so in current smoking COPD (S-COPD) ($P < 0.005$).

pSmad 7 expression (Fig 6&7):

In general, pSmad 7 expression was inverse of that for pSmad 2/3, but not always.

Epithelium:

In contrast to pSmad 2/3, pSmad 7 stained cells in the basal area of the epithelium, was slightly increased in NLFS ($P<0.01$) but slightly reduced though unchanged in the COPD groups.

Rbm:

There was little change in Rbm cell expression of pSmad 7, with just a slight increasing in NLFS and again a slight decrease in COPD.

The pattern in Rbm vessels was similar; through the decrease in percent vessel staining in S-COPD was much more marked.

LP:

In absolute numbers, there was a reduction in pSmad 7 cell expression in smokers and COPD about equally.

In LP vessels, there was a decrease in pSmad 7 expression in NLFS which was more marked and significant in COPD groups. In percentage terms, taking into account a reduction in vessels in COPD LP, the pattern was similar but with a more marked decrease in ES-COPD, and little change in normal lung function smokers i.e. more of a COPD-only effect.

pSmad 2/3:7 ratios for cellular expression (Fig 8)

In the epithelium, there was a significant increase in this ratio only in current smoker COPD (S-COPD) subjects.

In the Rbm there was a large increase in pSmad 2/3 to 7 ratio in both COPD groups. Finally, in LP cells there was again a marked increase in the ratio in both COPD groups (<0.001).

Regression Data (Fig 9 & 10)

A) pSmads basal cell expression versus lung function:

Right across the board, whether looking at FEV₁, FEV₁/FVC ratio (FER), or FEF₂₅₋₇₅ % (small airways), there were remarkably consistent negative relationships in all smokers/COPD groups with pSmad 2/3 cell expression, and positive relationships with pSmad 7 expression. This is illustrated for ES-COPD in figure 9. This group was chosen for presentation since it is less likely confounded by potential co-existing effects of active smoking in addition to COPD, though each pathological group data were very similar.

B) Basal cell pSmads expression versus basal cell S100A4 (EMT bio-marker):

In order to elaborate the relationship between expression of these Smad transcriptional pathways and EMT, cellular expression of both pSmad 2/3 and pSmad 7 were correlated with the classical EMT mesenchymal marker S100A4 (8). Again, there were consistent relationships between epithelial basal cell and Rbm cell expression of pSmad 2/3 (positive) and pSmad 7 (negative) with S100A4. Similarly

the pSmad 2/3:7 ratio also had consistently positive relationships with the EMT marker (Fig-10, with ES-COPD as the exemplar).

C) Basal cells TGFβ₁ expression against pSmads and S100A4 expression and lung function:

In contrast, there were no significant relationships shown in either of these regression analyses.

DISCUSSION

COPD is a potentially complex condition, but the basic pathophysiology at least in early disease is fibrosis and destruction of small airways (21). However, we do not understand why this process occurs apart from smoking being a driver of it. Thus, although COPD is usually described as an abnormal inflammatory immune response to noxious particles and gases, how that actually translates into airway obstruction is not understood (22). There is now good evidence that there is dysregulated epithelial basal stem cell function (23), one feature of which is active EMT (8). TGF β_1 has been suggested as a key regulator of the fibrotic airway disease in COPD (24). Although considerable progress has been made in general in understanding TGF β_1 and associated intracellular downstream pathways, little is known about how TGF β_1 is involved in the pathology of COPD. However, cigarette smoke has been shown to induce TGF β_1 release by cultured mouse tracheal explants (25), although its downstream effectors, the activated transcription factors Smad 2/3 were reported not to be increased in expression in a histochemical study of small airways in COPD (26). The same authors did report downregulation of Smad 7 expression (26). TGF β_1 has been postulated also as a key inducer of EMT in several tissues, including alveolar type II cells (27) in relation to idiopathic pulmonary fibrosis (IPF) (28), so some connection between TGF β_1 , EMT and fibrosis is appearing in lung pathology.

The activated receptor for TGF β_1 signals through phosphorylation of Smad transcription factors, known as its “canonical pathway”. However, as mentioned above it is not clear whether pSmads are active in the COPD airway wall or involved in inducing EMT or airflow obstruction in COPD. Ours is the first comprehensive

study to analyse the expression and assessment of the $TGF\beta_1$ -related “classical” pathway transcriptional proteins in airway tissues from normal smokers and patients with COPD compared to normal controls. We have studied this in large airways because previous investigations have shown especially active EMT in this compartment, although a similar but less marked picture was also found in small airways (9). We have previously reported $TGF\beta_1$ expression to be increased in the epithelial Rbm of smokers and especially those with COPD (18) but then we focused only on the relationship of $TGF\beta_1$ to vascular structures.

Our observation of an increase in $TGF\beta_1$ expression in basal cells of large airway epithelium in smokers is consistent with Takizawa and colleague’s observation that $TGF\beta_1$ expression is higher than normal in airway epithelial cells from COPD subjects and smokers (29). Similarly, De Boer et al found a small but significant increase in $TGF\beta_1$ expression in small airway epithelium from COPD patients (30), suggesting that our data may be generalizable to the whole airway tree. . In the current study the fact that cellular pSmad expression was closely associated in regressions with both EMT activity and airflow obstruction suggest strongly that this pathway is of functional significance in COPD pathogenesis.

Several genetic studies have demonstrated a link between COPD and $TGF\beta_1$ in COPD (31). $TGF\beta_1$ can also induce EMT- type changes in respiratory epithelial cells (32). The epithelium in smokers is also likely to produce more $TGF\beta_1$, suggesting the possibility of a self-reinforcing vicious cycle to EMT stimulation (33). Our finding is novel in that we have shown both a general increase in $TGF\beta_1$ in all airway wall compartments studied, but a parallel change in downstream pSmad signalling.

These changes were also evident in both smokers and COPD generally, but more so in actively smoking COPD. It is in line with previous studies where anti TGF β treatment was able to attenuate both airway pathology and emphysema in mice (34).

In our work, although TGF β_1 expression was increased in the smoker/COPD airways, unlike pSmad there was no relationship to either EMT activity or obstructive lung function. This suggests that other growth factors or change in receptor responsiveness to TGF β_1 are involved. The Wnt- β catenin pathway has been previously described as a mediator in pulmonary fibrosis (35) and other signalling pathways such as Notch, nuclear factor-kB and Hedgehog (Hh) have been also shown to participate in EMT in some circumstances (36). Further work on these pathways in COPD needs to be done.

Evidence of increased TGF β_1 expression by Rbm vessels was further supported by similar Rbm vascular expression of pSmad2/3 in smokers and COPD. Thus supports our previous suggestions that this system may be a driving factor for angiogenesis in the Rbm to produce the picture of Type-3 EMT we have described in large airways in COPD (9). Type-3 EMT is generally thought to be pre-malignant (37) A link between this process and malignancy is reinforced by the finding that EMT expression through a Smad-binding element is active in non-small cell lung carcinoma (38) where the TGF β -Smad system also facilitates metastasis (39). It needs to be noted that lung cancer development is especially evident in smokers who have also developed COPD (40).

The large majority of cells in the LP are thought to be stromal cells (41) and it is most likely that they are the one expressing Smad pathway activation. This would fit with

evidence that lung fibroblasts in COPD patients produce increased extracellular matrix (ECM), dependent on the local cytokine environment (42).

One of the most remarkable findings in our current study was the significant correlation of pSmad 2/3 and pSmad 7 expression with both an EMT activity marker and airflow obstruction. This “mechanistic” relationship gives our findings relevance for the core COPD pathophysiology. Interestingly, in more advanced disease and in small airways, Zandvoort and colleagues did not find pSmad 2 expression in COPD; it may be that our study into earlier disease in COPD was more appropriate for investigating pathogenic mechanisms (26).

The strengths of the present study include the use of relevant human tissue in very well phenotyped individuals with mild to moderate COPD, with fairly robust numbers giving sufficient power to detect these fascinating findings. This follows our previous work on EMT biomarker expression in both large and small airways. We have focused on mild to moderate COPD patients, because we wanted to look at pathogenic mechanisms in relatively early disease with relatively little confounding by chronic infection and emphysema. There are also a few limitations to this study. Firstly, it is cross-sectional at a single time point and longitudinal studies would be required to see how variable $TGF\beta_1$ -pSmad expression is within individuals and if it relates to the natural history of smoker’s airway disease as it becomes more severe. Secondly, we are not yet sure about the true phenotype of the of LP cells expressing pSmad which will be a future goal. Finally, our control subjects were somewhat younger than the smoker/COPD group, but there was no evidence that this was a confounder as the growth factor and transcription factor expression levels were not age-related across any group.

Other future goals for research should include investigating other transcriptional pathways and proteins that could contribute potentially to driving EMT, including especially β -Catenin, Twist and Snail-1, and emphasising the process involved in small airways in comparison to large airways, the former being where the main destructive pathophysiology occurs.

Conclusion & Summary:

In conclusion, the TGF β ₁-pSmad pathway is likely to be an important regulator of smoking-induced airway pathology including EMT, but also airway fibrosis leading to airway obstruction. The present study demonstrated a positive association between smoking, COPD and up regulation of stimulatory pSmads in all airway compartments, and in vessels as well as cells. The relationships seen between pSmad expression and EMT and also airflow obstruction supports these statements.

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Table 1. Demographic detail and lung function data for participants

Groups (numbers)	NC (n=15)	NLFS (n=15)	S-COPD (n=17)	ES-COPD (n=15)
GOLD I/GOLD II‡	N/A	N/A	10/7	8/7
Male/female	7/8	11/4	9/8	9/6
Age (years)	44 (20-68) (p=0.313)	50 (30-66) (p=0.313)	61 (46-78) (p=0.001)*	62 (53-69) (p=0.001)*
Smoking (pack years)	0	32 (10-57)	45 (18-78)	51 (18-150)
FEV ₁ % predicted (Post BD)†	113 (86-140)	99 (78-125) (p=0.01)*	83 (66-102) (p<0.001)*	83 (54-104) (p<0.001)*
FEV ₁ /FVC % (Post BD)†	82 (71-88)	77 (70-96) (p=0.218)	59 (46-68) (p<0.001)*	57 (38-68) p<0.001)*
Data expressed as median and range. NC- Normal control; NLFS-Normal lung function smoker; COPD-CS-COPD current smoker; COPD-ES- COPD ex-smoker; N/A-Not any				
*Significance difference from NC				
† Post BD values after 400µg of salbutamol				
‡ Diagnosis of COPD was made according to GOLD 2015 guidelines				

Legend

Fig-1: Transforming growth factor beta ($TGF\beta_1$) - Smad pathway

Fig-2: Comparison of $TGF\beta_1$ expression in A) basal epithelial cells B) Rbm cells and C) Rbm vessels, between healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

Fig-3: Representative photomicrograph of $TGF\beta_1$ expression in 4 different study group.

A) Healthy non-smokers (H-N), B) smokers with normal lung function (NLFS), C) current smoking COPD (S-COPD) and D) ex-smokers with COPD (ES-COPD). Original magnification, $\times 400$. Scale bar = 50 μm .

Fig-4: Comparison of phosphorylated (p) Smad 2/3 expression in A) basal epithelial cells B) Rbm cells, C) Rbm vessels, D) LP cells, E) as % of LP cells stained and F) as number of LP vessels stained.

Fig-5: Photomicrographs of pSmad 2/3 expression representing 4 different study groups:

A) Healthy non-smokers (H-N), B) smokers with normal lung function (NLFS), C) current smoking COPD (S-COPD) and D) ex-smokers with COPD (ES-COPD). Original magnification, $\times 400$. Scale bar = 50 μm .

Fig-6: Comparison of phosphorylated (p) Smad 7 expression in A) basal epithelial cells B) Rbm cells and C) LP cells. D) LP vessels, in healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

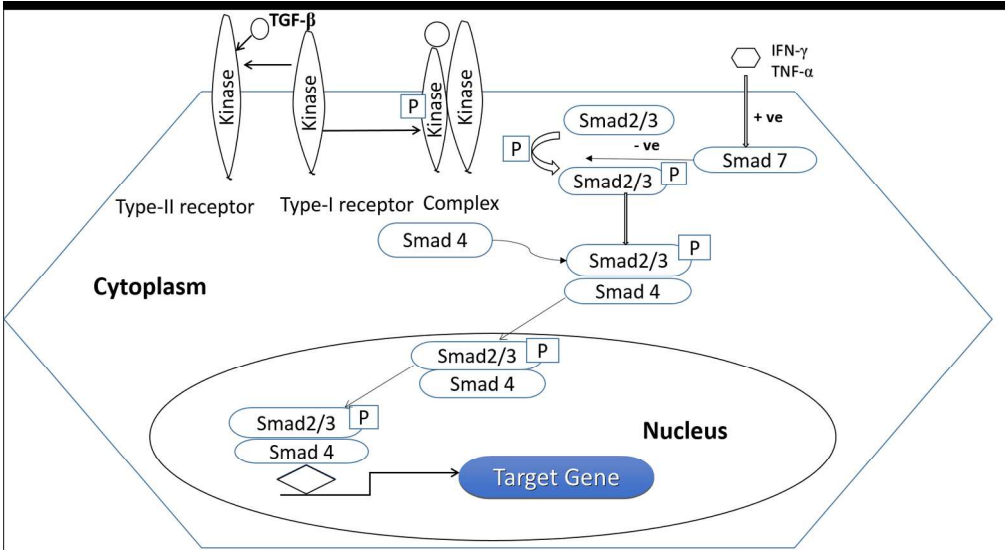
Fig-7: Representative photomicrograph of pSmad 7 expression in 4 different study groups:

A) Healthy non-smokers (H-N), B) smokers with normal lung function (NLFS), C) current smoking COPD (S-COPD) and D) ex-smoker with COPD (ES-COPD). Original magnification, $\times 400$. Scale bar = 50 μm .

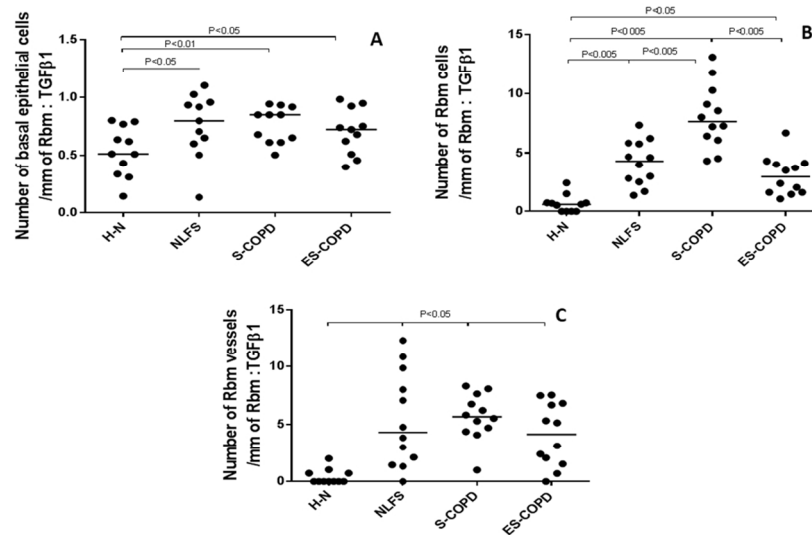
Fig-8: Comparison of cellular expression of pSmad 2/3:7 ratio in A) basal epithelial cells B) Rbm cells and C) LP cells of healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

Fig-9: A) Correlation between number of basal epithelial cells positive for pSmad 2/3 and forced expiratory ratio (FER); (B) The same for % predicted FEF_{25-75} (an index of small airway calibre); (C) Correlation between pSmad 7 positive basal epithelial cells and $\text{FEF}_{25-75}\%$ predicted; (D) Correlation between basal epithelial cell pSmad 2/3:7 ratio with forced expiratory volume in 1second (FEV_1). All these correlations are for the ex-smoker with COPD group (ES-COPD). For pSmad 2/3 there is much higher expression with low lung function and increased obstruction, and vice versa with pSmad 7.

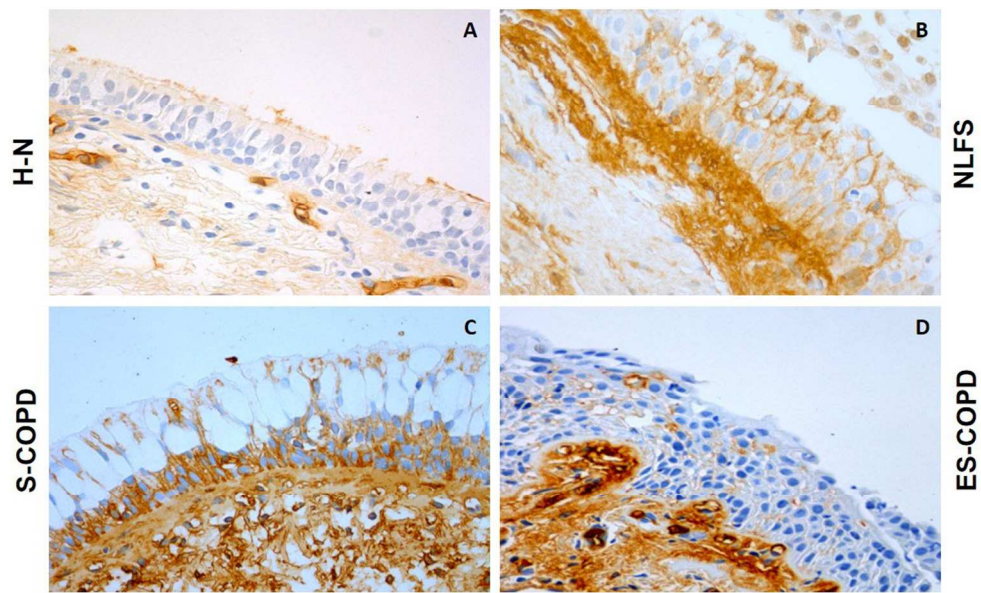
Fig-10: (A) Correlation between number of Rbm cells positive for pSmad 2/3 with S100A4 positive Rbm cells; (B) Correlation between number of basal epithelial cells positive for pSmad 7 with S100 positive basal cells; (C) Correlation between basal epithelial pSmad 2/3:7 ratio with S100A4 positive basal epithelial cells All these correlation are also from the ex-smoker COPD group (ES-COPD).



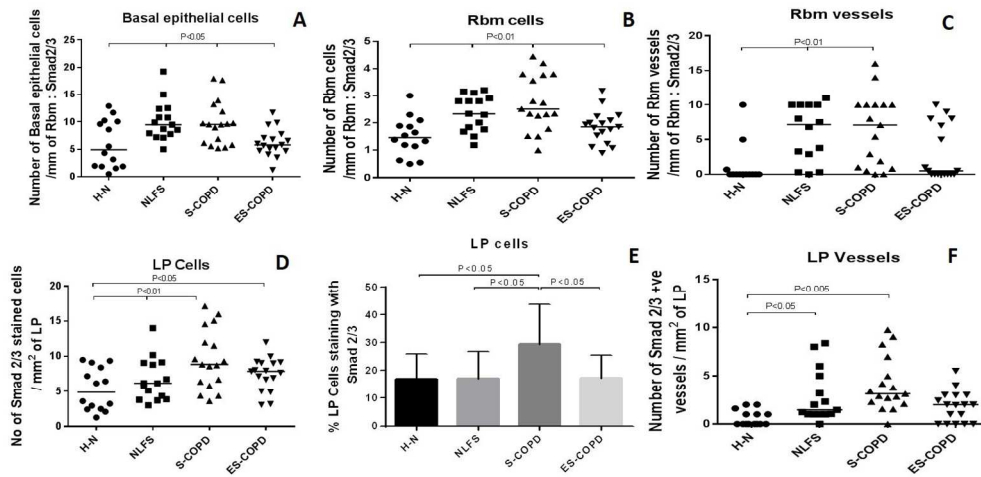
Transforming growth factor beta (TGFβ1) - Smad pathway
163x89mm (300 x 300 DPI)



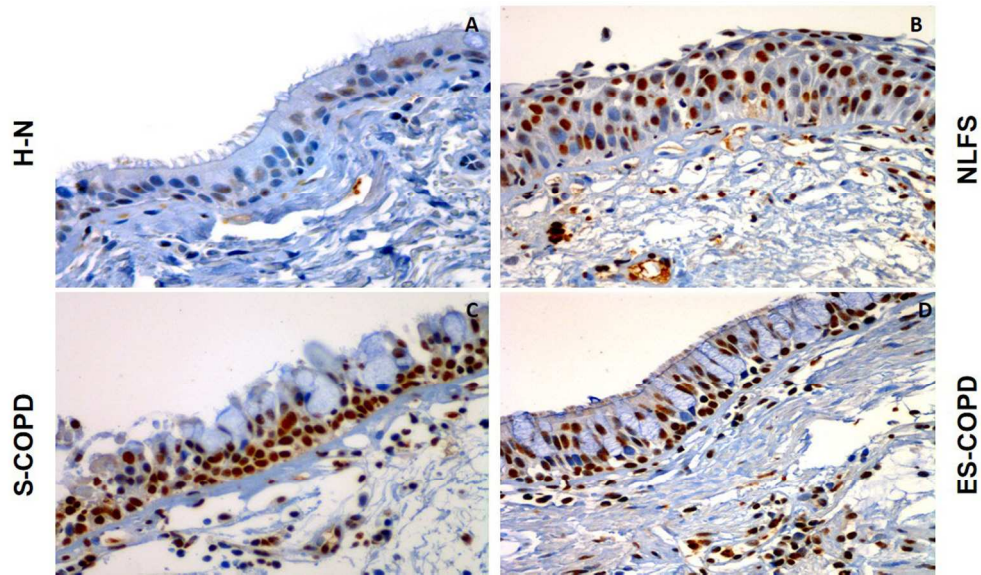
Comparison of TGFβ1 expression in A) basal epithelial cells B) Rbm cells and C) Rbm vessels, between healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).
108x60mm (300 x 300 DPI)



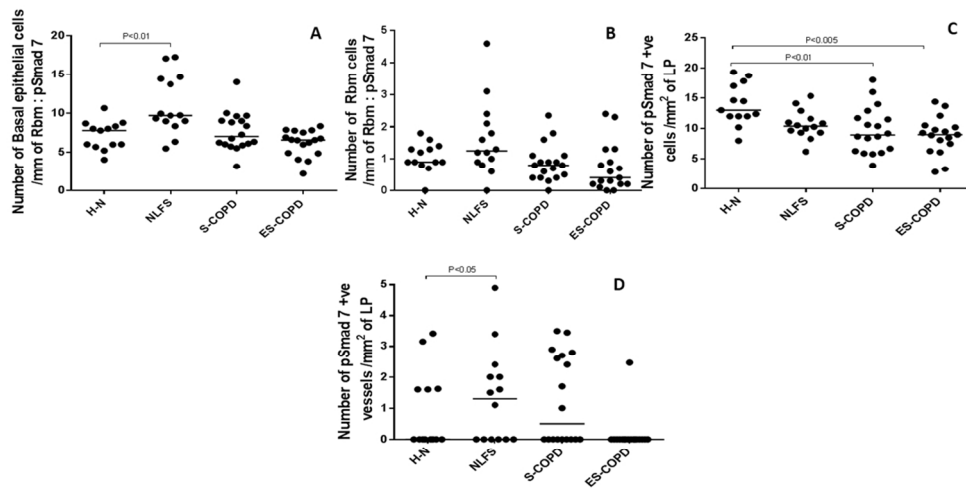
Representative photomicrograph of TGFβ1 expression in 4 different study group.
A) Healthy non-smokers (H-N), B) smokers with normal lung function (NLFS), C) current smoking COPD (S-COPD) and D) ex-smokers with COPD (ES-COPD). Original magnification, ×400. Scale bar =50 μm.
104x60mm (300 x 300 DPI)



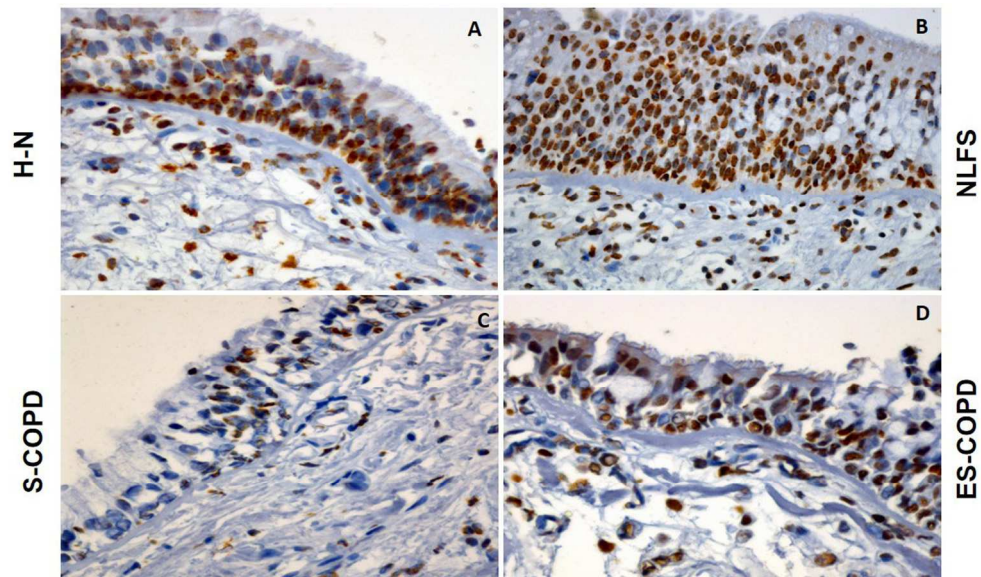
Comparison of phosphorylated (p) Smad 2/3 expression in A) basal epithelial cells B) Rbm cells, C) Rbm vessels, D) LP cells, E) as % of LP cells stained and F) as number of LP vessels stained. 140x68mm (300 x 300 DPI)



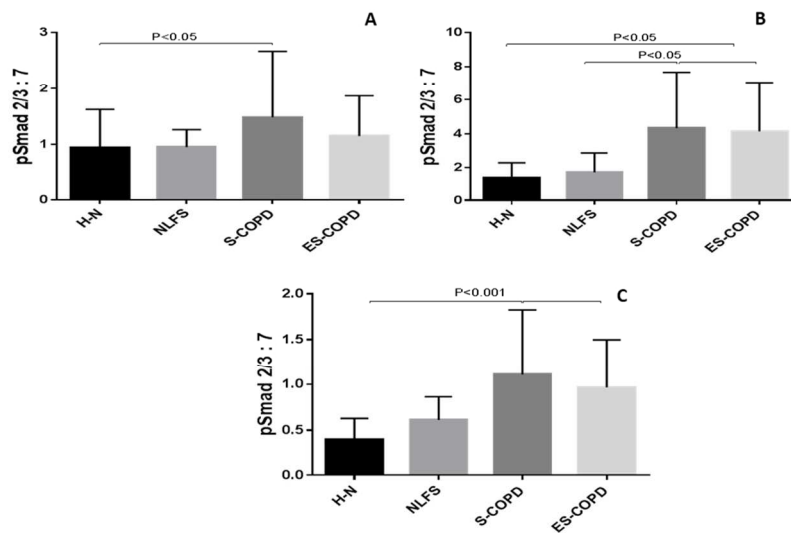
Photomicrographs of pSmad 2/3 expression representing 4 different study groups:
A) Healthy non-smokers (H-N), B) smokers with normal lung function (NLFS), C) current smoking COPD (S-COPD) and D) ex-smokers with COPD (ES-COPD). Original magnification, $\times 400$. Scale bar = 50 μm .
105x60mm (300 x 300 DPI)



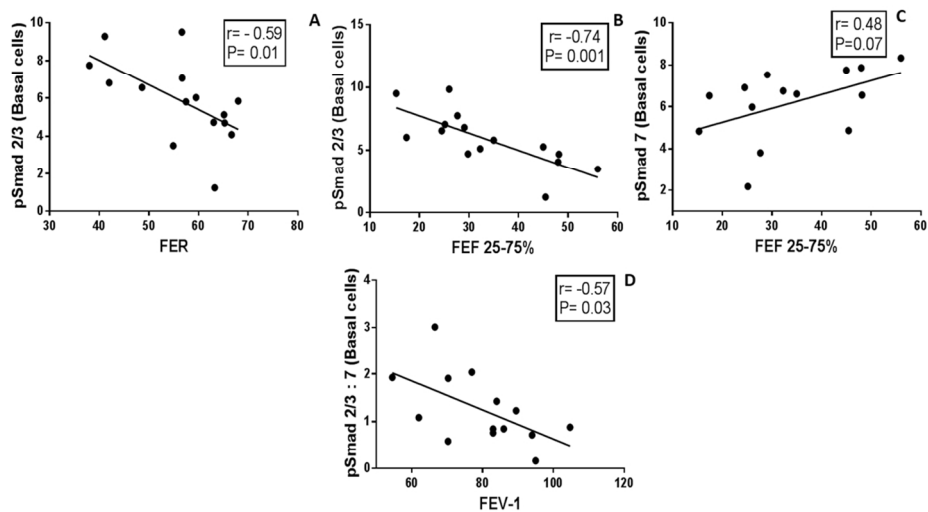
Comparison of phosphorylated (p) Smad 7 expression in A) basal epithelial cells B) Rbm cells and C) LP cells. D) LP vessels, in healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).
108x60mm (300 x 300 DPI)



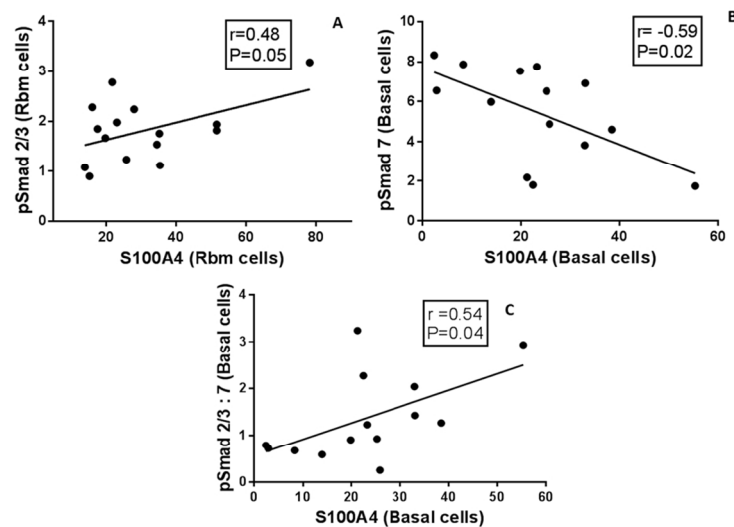
Representative photomicrograph of pSmad 7 expression in 4 different study groups:
A) Healthy non-smokers (H-N), B) smokers with normal lung function (NLFS), C) current smoking COPD (S-COPD) and D) ex-smoker with COPD (ES-COPD). Original magnification, $\times 400$. Scale bar = 50 μm .
105x60mm (300 x 300 DPI)



Comparison of cellular expression of pSmad 2/3:7 ratio in A) basal epithelial cells B) Rbm cells and C) LP cells of healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).
108x60mm (300 x 300 DPI)



A) Correlation between number of basal epithelial cells positive for pSmad 2/3 and forced expiratory ratio (FER); (B) The same for % predicted FEF25-75 (an index of small airway calibre); (C) Correlation between pSmad 7 positive basal epithelial cells and FEF 25-75% predicted; (D) Correlation between basal epithelial cell pSmad 2/3:7 ratio with forced expiratory volume in 1second (FEV1). All these correlations are for the ex-smoker with COPD group (ES-COPD). For pSmad 2/3 there is much higher expression with low lung function and increased obstruction, and vice versa with pSmad 7.
108x60mm (300 x 300 DPI)



(A) Correlation between number of Rbm cells positive for pSmad 2/3 with S100A4 positive Rbm cells; (B) Correlation between number of basal epithelial cells positive for pSmad 7 with S100A4 positive basal cells; (C) Correlation between basal epithelial pSmad 2/3:7 ratio with S100A4 positive basal cells. All these correlations are also from the ex-smoker COPD group (ES-COPD).
108x60mm (300 x 300 DPI)